

Early Life Exposure to Undernutrition Induces ER Stress, Apoptosis, and Reduced Vascularization in Ovaries of Adult Rat Offspring¹

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ABSTRACT

Maternal nutritional restriction has been shown to induce impairments in a number of organ systems including the ovary. We have previously shown that maternal undernutrition induces fetal growth restriction and low birth weight, and results in an offspring ovarian phenotype characteristic of premature ovarian aging with reduced ovarian reserve. In the present study, we set out to investigate the underlying mechanisms that lead offspring of undernourished mothers to premature ovarian aging. Pregnant dams were randomized to 1) a standard diet throughout pregnancy and lactation (control), 2) a calorie-restricted (50% of control) diet during pregnancy, 3) a calorie-restricted (50% of control) diet during pregnancy and lactation, or 4) a calorie-restricted (50% of control) diet during lactation alone. The present study shows that early life undernutrition-induced reduction of adult ovarian follicles may be mediated by increased ovarian endoplasmic reticulum stress in a manner that increased follicular apoptosis but not autophagy. These changes were associated with a loss of ovarian vessel density and are consistent with an accelerated ovarian aging phenotype. Whether these changes are mediated specifically by a reduction in the local antioxidant environment is unclear, although our data suggest the possibility that ovarian melatonin may play a part in early life nutritional undernutrition and impaired offspring folliculogenesis.

apoptosis, ER stress, follicles, maternal nutrition, ovary, reproduction

INTRODUCTION

Early life events, including changes in nutritional status, trigger processes that prepare the individual for particular circumstances that are anticipated in the postnatal environment. However, where the intrauterine and postnatal environments differ markedly, such changes to the developmental trajectory may prove maladaptive in later life [1–5]. Research into the developmental programming of disease risk has integrated aspects of metabolic, cardiovascular, and reproductive physiology, and evidence indicates that reproductive maturation and function is similarly influenced by early life events, which has been established in human populations [6–12]. In experimental models, the impact of early life nutritional adversity on reproductive function has been documented by our group [13–15] and others [16–20]. We have previously reported that rat offspring born to undernourished mothers have reduced ovarian follicle numbers and increased ovarian oxidative stress as adults [13]. Others have shown consistent effects where maternal malnutrition during lactation impairs folliculogenesis in rat offspring at puberty [21] and in sheep results in an increase in apoptosis-regulating genes [22]. Although evidence for early life nutritional influences on reproductive function is mounting, the signaling pathways that elicit ovarian impairment after early life undernutrition are still poorly understood.

We have shown previously that fetal growth restriction in the rat following maternal undernutrition results in advancements in pubertal onset [14] in a manner similar to that reported in low birth weight girls [6, 7, 23]. Offspring of undernourished mothers also demonstrated reductions in primordial, secondary, and antral follicles as adults [13] in a manner that was dependent upon the timing of undernutrition. Importantly we have shown that pregnancy is the critical window of fetal ovarian vulnerability, where offspring of mothers undernourished during pregnancy and lactation or pregnancy alone, but not during lactation alone, demonstrated a loss of oocyte-derived follicular growth factors as well as increased oxidative stress levels [13]. Previous studies have shown in vitro that ovarian oxidative stress induces granulosa cell apoptosis and subsequently follicle atresia in preovulatory rat follicles [24]. These data are characteristic of premature ovarian aging [25] and are similar to our observations in offspring born to undernourished mothers [13].

Thus, in the present study, we set out to investigate the underlying mechanisms that lead offspring of undernourished mothers to premature ovarian aging. We determined potential signaling molecules responsible for our observed increase in oxidative stress and loss of ovarian reserve [13]. In doing so, we concentrated on signaling pathways known to be stimulated

¹D.M.S. is supported by Canada Research Chair in Perinatal Programming, J.J.P. is supported by Canadian Institutes for Health Research, and M.H.V. is supported by Gravida. This study was in part funded by the Health Research Council of New Zealand, and Gravida. Presented in part at the 47th Annual Meeting of the Society for the Study of Reproduction, 19–23 July 2014, Grand Rapids, Michigan.

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by the accumulation of reactive oxygen species, including endoplasmic reticulum (ER) stress [26], phosphoinositide 3-kinase (PI3K) [27], and melatonin [28]. We hypothesized that increased ovarian ER stress could be associated with follicular loss through apoptosis and/or autophagy [29] and that this could be PI3K-inflammation mediated. Ovarian aging is associated with decreased vascularization and ovarian stromal blood flow [30]. Compromised perfollicular vascularization has been proposed to contribute to an accumulation of oxidative stress in aging follicles [31], and altered vascular support may have long-term effects on follicle depletion [32]. Thus, we investigated whether early life undernutrition altered ovarian levels of a known angiogenic agent vascular endothelial growth factor (VEGF), its receptor (VEGFR2), and blood vessel density. Because VEGF and VEGFR2 binding has been suggested to protect cells from apoptosis [33], we anticipated a decrease in VEGF in offspring ovaries accompanied by a loss in ovarian vascularization.

Melatonin induces ovarian cell survival [34] and is a powerful antioxidant and free radical scavenger [35], reducing atresia [34] and improving in vitro fertilization rates [36] and oocyte quality [37]. In brain tissue, melatonin induces protein kinase B (Akt) phosphorylation [38] because its receptors induce PI3K signaling [39]. We were interested in investigating whether the capacity to produce ovarian melatonin was diminished in undernourished offspring, implicating this important hormone in adult ovarian oxidative stress and potential follicle loss. Because melatonin and circadian locomotor output cycles kaput (Clock) genes share a close relationship [40] and recent work has suggested that clock genes are altered in offspring ovaries after early life nutritional manipulation [41], we also determined whether early life undernutrition altered adult ovarian clock gene levels.

MATERIALS AND METHODS

Animal Model

We used an established model of global maternal nutrient manipulation [13, 14, 42] to induce fetal growth restriction. Briefly, female Wistar rats (aged 120 days) were time mated, and after confirmation of mating, rats were housed individually in standard rat cages with free access to water. All rats were kept in the same room with a constant temperature maintained at 25°C and a 12L:12D cycle. Pregnant dams were randomized into one of four groups: 1) dams fed a control diet of 18% protein, 5% fat, and 3.4 kcal/gm digestible energy (Diet 2018; Harlan Teklad) ad libitum throughout pregnancy and lactation (Cont; n = 8); 2) undernourished dams fed 50% of Cont intake throughout pregnancy and lactation (UNPL, n = 7); 3) undernourished dams fed 50% of Cont intake throughout pregnancy only (UNP, n = 6); and 4) undernourished dams fed 50% of Cont intake throughout lactation only (UNL, n = 6). After birth, the pups were sexed, weighed, and litter size was standardized to eight pups that best represented (were closest to) the mean litter weight. Each litter contained four females and four males. At weaning (Day 22), the offspring were weighed, and female offspring were weight-matched within maternal dietary groups, housed two per cage, and fed the control diet ad libitum for the remainder of the study. At 150 days of age, offspring (from different litters) were fasted overnight and killed by decapitation following pentobarbitone anesthesia (60 mg/kg, subcutaneously). All the ovarian tissue was collected between 0900 and 1200. While under anesthesia, vaginal smears were performed for determination of estrous stage [13–15]. Both ovaries were collected; one was fixed in Bouin solution (Sigma-Aldrich) and processed for the determination of follicular counts [13] or for immunohistochemistry and the other snap-frozen and stored at –80°C for molecular analyses. In all cases, all biological replicates are from different litters. All the animal experiments were approved by the Animal Ethics Committee, University of Auckland (Approval R402).

Molecular Analyses

RNA extraction and reverse transcription. Ovarian tissues were collected, snap-frozen, and stored at –80°C in preparation for RNA extraction and reverse transcribed as previously described [13, 43, 44]. Total RNA was extracted

using AllPrep DNA/RNA Mini kit (80204; Qiagen). Genomic DNA was removed with RNase-free DNase (Invitrogen Life Technologies). RNA quantity and purity were analyzed using a NanoDrop spectrophotometer (ND-1000; BioLab Ltd.) and NanoDrop software (version 3.1.2). RNA samples were stored at –80°C.

Five micrograms of total RNA was used for first-strand cDNA synthesis using the Moloney Murine Leukemia Virus Reverse Transcriptase enzyme (M-MLV-RT) (Promega Corp.) and a standard thermocycler (GeneAmp PCR System 9700; Applied Biosystems). A master mix was prepared containing 5 ml M-MLV 56 buffer (M531A; In Vitro Technologies), 0.5 ml M-MLV-RT (M170B; In Vitro Technologies), and 1.25 ml 10 mM deoxyribonucleotides (R0181; Global Science) under the following cycling conditions: denaturation stage of 5 min at 96°C, followed by 30 cycles of 30 sec each of 96°C (denaturation), 60°C (annealing stage), and 72°C (extension stage). Complementary DNA was stored at –20°C.

Quantitative polymerase chain reaction assays. A quantitative PCR assay was performed as previously described [13] using the Roche LightCycler 480 System (Roche Diagnostics) and LightCycler 480 SYBR Green I Master (04707516001; Roche Diagnostics). Primers for all genes for ER stress, autophagy, inflammation, gonadotropin receptor, and clock genes—except Period and hydroxyindole O-methyltransferase (HIOMT)—were designed using Primer BLAST software available at the National Center for Biotechnology Information Web site (blast.ncbi.nlm.nih.gov; Table 1). Primers were manufactured by Invitrogen (Invitrogen Life Technologies). Ready-made primers for Period (per1, per2) and HIOMT genes were purchased from Qiagen (Table 2).

Optimal primer conditions were adjusted to the following cycling conditions: length = 20 bp (range 17–23 bp), T_m = 63°C (range 60°C–65°C), and amplicon length = 100–350 bp. Dissociation analyses were performed to ensure specificity, and samples producing a single peak in dissociation curves were used. A subset of amplified products was visualized on an agarose gel using the E-GelH CloneWell 0.8% SYBR Safe gel (G6618-08; Invitrogen) run on the E-GelH iBase™ Power System (G6400; Invitrogen).

All the quantitative PCRs were carried out with an initial denaturation at 95°C for 5 min followed by amplification of the gene product through 45 successive cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec. A standard curve was generated from the mean cycle threshold of eight standards (1:5 serial dilution) of a known concentration in triplicate, while amplification and dissociation curves were generated for all standards and samples (Roche Lightcycler 480 System; Roche Diagnostics). Each sample was run in triplicate. All the mRNA data is expressed relative to the geometric mean of two different reference genes (cyclophilin and beta-actin), the levels of which did not differ between nutritional groups.

Immunohistochemistry and Immunofluorescence

Fixed ovaries were processed for microscopy, embedded in paraffin, and the entire ovary was serially sectioned at 8 μm as described previously [13]. Sections that were not used for follicle counts were then used for immunohistochemistry/immunofluorescence.

Determination of Follicular Apoptosis

Detection of apoptotic cells in follicles was performed as previously described [33]. A terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) kit (Roche Applied Science) was used according to the manufacturer's protocol. Briefly, ovarian tissue sections (8 μm) were permeabilized in 0.1% Triton X-100 (Sigma), washed in PBS, and incubated for 60 min with the fluorescein isothiocyanate-conjugated TUNEL enzyme to detect DNA fragmentation. Nuclei were counterstained with propidium iodide. Apoptotic cells showing TUNEL-positive staining were counted in six fields of view per section at 250× magnification (n = 5 per nutritional group). Image analysis was performed using an Olympus BX-61 microscope and integrated morphometry software (MetaMorph). Data are expressed as apoptotic-positive follicular cells as a proportion of the total.

Determination of Ovarian Blood Vessel Density and Immunolocalization of VEGF and VEGFR2

Evaluation of blood vessel density was performed as described previously using the endothelial cell marker, CD31 [45]. Briefly, ovarian sections (8 μm) were immersed in 30% hydrogen peroxide to inhibit endogenous peroxidase activity for 10 min. Antigen retrieval was performed by incubating tissues in 10 mM sodium citrate buffer with Tween, pH 6.0, at 90°C for 12 min. Nonspecific

TABLE 1. Primer sequences.

Rat gene ^a	Forward primer	Reverse primer	Amplicon length (bp)	GenBank accession no.
<i>Beta-actin</i>	CACCAACTGGGACGATATGGA	CAGCCTGGATGGCTACGTACAT	188	NM_031144
<i>Cyclophilin</i>	TTGGGTGCGCTCTGCTTCTCGA	GCCAGGACCTGTATGCTTCA	240	NM_017101.1
<i>BMAL1</i>	ACTGCACCTCGGGAGCGACT	CGCCCGATTGCAACGAGGCA	320	NM_024352.2
<i>CLOCK</i>	ACCGCACCTGCCAGTTCATG	GCGTGTCCGCTGCTTAGCT	214	NM_021856.1
<i>CRY1</i>	CGGAAGCTCGTGTCCGGTCCG	CGCGCGACGTCCTTCAGGAG	232	NM_198750.2
<i>CRY2</i>	ACGGTCCCGCTGCAGTCGAT	CTGACGAGGAGGCCCGCAAC	166	NM_113405
<i>p21</i>	AGCCACAGGCACCATGTCCGA	CGCATCGCAATCGCGGCTCA	118	U24174.1
<i>XBPs</i>	GAGTCCCGCAGGTTG	GCGTCAGAATCCATGGA	165	NM_001004210
<i>XBP1t</i>	GAGCAGCAAGTGGTGGATTT	TCTCAATCACAAGCCCATGA	197	NM_001004210
<i>P13Kca (p110)</i>	GAACGTGTGCCGTTTGTTTT	ACCATGATGTGCGTCATTCA	300	NM_133399.2
<i>P1K3r1 (p85)</i>	AGCAACCGAAACAAAGCCGA	ATAGCCGGTGGCAGTCTTGT	153	NM_013005.1

^a *BMAL1*, brain and muscle Arnt-like protein 1; *CLOCK*, circadian locomotor output cycles kaput; *P13Kca (p110)*, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; *CRY1* and *CRY2*, cryptochrome 1 and 2, respectively; *P1K3r1 (p85)*, phosphatidylinositol 3-kinase regulatory subunit 1 (alpha); *p21*, cyclin-dependent kinase inhibitor 1 (also known as CDK-interacting protein 1); *XBPs* and *XBP1t*, X-box binding protein 1 spliced and total, respectively.

binding was blocked with 5% bovine serum albumin for 10 min at room temperature (RT) and incubated overnight at 4°C with anti-CD31 primary antibody (1:100 dilution; Abcam). The following day, tissue sections were incubated at RT for 2 h with anti-mouse biotinylated secondary antibody (Sigma-Aldrich Canada Ltd.) then incubated with ExtrAvidin (Sigma-Aldrich Canada Ltd.) for 1 h. CD31 was visualized with 3,3'-diaminobenzidine (DAB) (D4293; Sigma-Aldrich), and all sections were counterstained with Gills 2 hematoxylin (GHS216; Sigma-Aldrich). For blood vessel density determination, CD31-immunopositive (endothelial cells) blood vessels were measured in six fields of view per section at 250× magnification (n = 5 per nutritional group). Image analysis was performed using an Olympus BX-61 microscope and integrated morphometry software (MetaMorph). Data are expressed as vessel area as a proportion of total ovarian area analyzed.

Protein localization of vascular endothelial growth factor (VEGF), its receptor type 2 (VEGFR2), and their colocalization was performed using immunofluorescence on ovarian sections (8 µm). Sections were incubated for 1 h with rabbit polyclonal anti-human VEGF (1:600 dilution; Santa Cruz Biotechnology), washed with PBS, and incubated for 1 h with an Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG; heavy and light chains), F(ab')₂ fragment (1:250 dilution, 8889; Cell Signaling). Sections were then washed twice for 2 min with 1× PBS and incubated with rabbit polyclonal anti-human VEGFR2 (1:500 dilution; Santa Cruz Biotechnology). After a 1-h incubation, tissue sections were washed with PBS and incubated for another hour with Alexa Fluor 594-conjugated anti-rabbit IgG (heavy and light chains), F(ab')₂ fragment. Nuclei were counterstained with propidium iodide or 4',6-diamino-2-phenylindole, and specimens were imaged with a BX-61 fluorescent microscope (Olympus). Stained slides were stored at 4°C. Total VEGF and VEGFR2 immunostaining was quantified by determining the proportion of immunopositive cells in six fields of view per section at 250× magnification (n = 5 per nutritional group). Image analysis was performed using an Olympus BX-61 microscope and integrated morphometry software (MetaMorph). Data are expressed as VEGF/VEGFR2-positive cells as a proportion of total ovarian area analyzed. The amount of area where VEGF and VEGFR2 colocalized was also calculated and expressed as a proportion of total area analyzed. All the image analyses were performed by an investigator blind to the study groups.

TABLE 2. Qiagen QuantiTect primer assays.

Rat gene ^a	Catalogue number	GenBank accession number
<i>PER1</i>	QT01615726	NM_001034125
<i>PER2</i>	QT00184737	NM_031678
<i>HIOMT</i>	QT02336901	NM_144759
<i>Beclin1</i>	QT00176344	NM_001034117
<i>NFκB</i>	QT01580012	NM_199267
<i>MAP1LC3a</i>	QT00371546	NM_199500
<i>IL-6</i>	QT00182896	NM_012589
<i>IL-1β</i>	QT00181657	NM_031512

^a *PER1* and *PER2*, Period 1 and 2 genes, respectively; *NFκB*, nuclear factor kappa-light-chain-enhancer of activated B cells; *IL-6*, interleukin 6; *IL-1β*, interleukin 1-beta; *MAP1LC3a*, microtubule-associated protein 1 light chain 3 alpha; *HIOMT*, hydroxyindole O-methyltransferase.

Immunolocalization of the Autophagy Regulator, Beclin1

Immunolocalization of Beclin1 in ovarian tissue sections was performed as described above with the following modifications. Ovarian sections were incubated with Beclin1 antibody (1:300 dilution, ab55878; Cedarlane) overnight at 4°C. The following day, sections were incubated with biotinylated secondary antibody (1:10 dilution, Vectastain Elite ABC kit peroxidase rabbit IgG; Vector Labs) for 2 h at RT then incubated with avidin-biotin peroxidase complex solution (4:10 dilution using 1% bovine serum albumin in 1× PBS) for 2 h at RT. The antibody complex was visualized using DAB, and the sections were counterstained with Gills II hematoxylin. Cover slips were mounted using Permount (SP15-500; Fisher Scientific), and immunopositive cells were imaged using the NIS-Elements AR imaging program on a Nikon 90i microscope at 200× magnification. Total Beclin1 immunostaining was quantified by determining average density of brown (DAB substrate) immunopositive cells in six fields of view per section at 200× magnification (n = 5 per nutritional group) using the NIS-Elements AR imaging program (Nikon). In all the immunohistochemical assays, negative controls were incubated in the absence of the primary antibody and were included in each assay run. All the image analyses were performed by an investigator blinded to the study groups.

Statistical Analyses

All the data were analyzed by one-way ANOVA. Data that were not normally distributed were log-transformed to achieve normality. Post-ANOVA multiple comparisons among means were performed using Bonferroni post hoc analysis where appropriate, and a *P* < 0.05 was considered statistically significant. All the data are presented as means ± SEM. Statistical analysis was performed using SigmaStat for Windows version 2.03 (Jandel Corp.) and GraphPad Prism version 6.00 for Mac (GraphPad Software).

RESULTS

Body Weights

At birth, sex ratios were the same across groups (data not shown). Early life undernutrition significantly reduced birth weight in UNP and UNPL offspring compared to Cont; this reduction in body weight persisted to weaning, and in UNPL, it persisted to adulthood [13] (Table 3). We found no effect of early life undernutrition exposure on adult ovarian weight [13].

Early Life Undernutrition Exposure Increased Ovarian ER Stress and Follicular Apoptosis but Decreased Ovarian Autophagy in Adult Offspring

Ovarian X-box binding protein 1 spliced (XBPs), total (XBP1t), and their ratio were determined as a marker of ER stress in offspring ovaries. Ovarian XBPs:XBP1t mRNA ratios were significantly increased in ovaries from offspring born to undernourished mothers (*P* < 0.001) (Fig. 1A). Post

TABLE 3. Offspring body and ovarian weights.*

Nutritional group	Birth weight (g)	Weaning weight (g)	Adult body weight (g)	Adult ovarian weight (g)
Control	6.1 ± 0.09 ^a	59.6 ± 0.7 ^a	301 ± 4.9 ^a	0.103 ± 0.005
UNP	4.8 ± 0.06 ^b	54.8 ± 1.2 ^b	292.4 ± 7.1 ^a	0.096 ± 0.005
UNPL	4.8 ± 0.08 ^b	32.7 ± 1.3 ^b	263.3 ± 6.6 ^b	0.097 ± 0.006
UNL	6.0 ± 0.08 ^b	39.7 ± 1.2 ^b	292.6 ± 15.6 ^a	0.100 ± 0.006

* Data are presented as mean ± SEM. Data are derived from Bernal et al. [13].

^{a,b} Values with differing letters are significantly different from one another, $P < 0.001$.

hoc analysis demonstrated that although UNP and UNPL offspring both showed an increased XBP1s:XBP1t ratio compared to Cont offspring, but only UNPL reached statistical significance (UNPL; $P < 0.001$) (Fig. 1A). Follicular apoptosis was significantly increased in ovaries from offspring born to undernourished mothers ($P < 0.001$) (Fig. 1B). Post hoc analysis demonstrated a statistically significant increase in the proportion of apoptotic follicular cells in ovaries of UNP ($P < 0.05$), and UNPL ($P < 0.05$) groups compared to Cont offspring (Fig. 1B).

Early life undernutrition exposure resulted in a significant decrease in adult ovarian mRNA levels of Beclin1 ($P < 0.001$), an established component of the autophagic machinery, and of the autophagosome marker LC3 ($P < 0.001$; Fig. 1C). Post hoc analyses showed a statistically significant decrease in Beclin1 mRNA levels in UNP ($P = 0.005$) and UNPL ($P < 0.001$) offspring compared with Cont offspring (Fig. 1D). Although both UNP and UNPL groups showed lower levels of LC3 mRNA, post hoc analyses showed a statistically significant decrease in LC3 mRNA levels only in UNPL offspring ($P < 0.001$; UNP: $P = 0.06$) (Fig. 1C). Ovarian sections were immunostained for Beclin1 protein to determine follicular localization. We observed that Beclin1 protein is localized in ovarian blood vessels, oocytes, and ovarian stroma (Fig. 1E). Consistent with mRNA levels, we observed a decrease in the mean density of immunostaining of Beclin1 protein in ovarian sections in UNP ($P < 0.001$), and UNPL ($P < 0.05$) groups compared to the Cont group (Fig. 1, E and F). UNL offspring showed similar staining to controls (Fig. 1, E and F). Immunopositive Beclin 1 was highly localized to oocytes and blood vessels and less so in stromal, granulosa, and thecal cells. All the negative controls in immunohistochemical staining assays showed no positive staining (data not shown).

Early Life Undernutrition Exposure Reduced Ovarian Blood Vessel Density and VEGF and VEGFR2 Immunostaining in Offspring Ovaries

Early life events have been shown to modulate vascularization of organs in offspring [46, 47]; thus, we set out to investigate whether blood vessel density was similarly altered in the ovary. Early life undernutrition exposure significantly decreased ovarian vascularization in a manner that was dependent upon timing of the undernutrition. Blood vessel density, including perfollicular capillaries, as defined by the presence of endothelial cell marker CD31 immunostaining as a proportion of total area, was significantly decreased in UNP ($P < 0.05$) and UNPL ($P < 0.05$) offspring compared to Cont

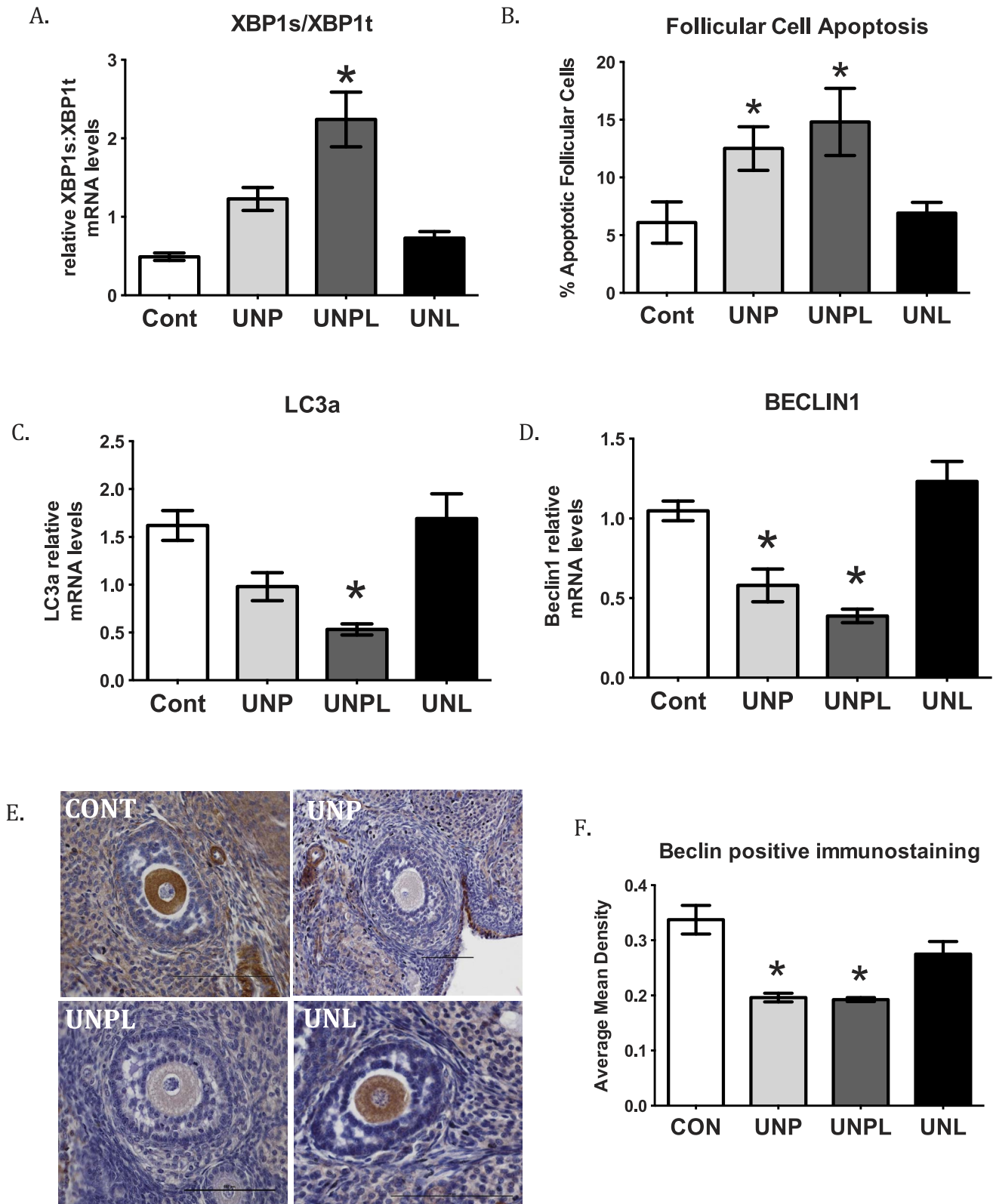
(Fig. 2). Vascularization in UNL offspring was not different to that of the Cont group.

VEGFR2 has been found to be highly expressed in stromal, granulosa, and theca cells in the rat ovary [48], and critical changes in sensitivity of granulosa cells to VEGF appear to be mediated through VEGFR2 [49]. VEGFR2 has also been shown not only to mediate vascularization, but also to function as an antiapoptotic agent in rat granulosa cells and is necessary for dominant follicle selection. Consistent with our observed increase in apoptosis and loss of vessel density, early life nutrient restriction resulted in a significant reduction in VEGF and VEGFR2 immunopresence in adult ovaries (Fig. 3). Immunohistochemical staining showed a significant decrease in VEGF and VEGFR2 protein in stromal and follicular cells as a proportion of total area analyzed in UNP ($P < 0.05$) and UNPL ($P < 0.05$) offspring compared to Cont offspring. Similarly, offspring born to undernourished mothers demonstrated decreased VEGF and VEGFR2 colocalization in UNP ($P < 0.05$) and UNPL ($P < 0.05$) offspring compared to Cont offspring (Fig. 3). All negative controls showed no positive staining (data not shown).

Early Life Undernutrition Exposure Altered Proinflammatory Cytokines and Upstream Signaling Molecules in Offspring Ovaries

We have previously reported that offspring exposed to early life undernutrition demonstrate increased levels of ovarian oxidative stress [13], and because we now demonstrated an increased in a marker of ER stress, we therefore investigated a central signaling pathway that could contribute to these observed changes. Offspring born to undernourished mothers demonstrated elevated mRNA levels of the genes that encode both the catalytic subunit (p110 α) and the regulatory subunit (p85 α) of PI3K, although only p110 α reached statistical significance ($P < 0.001$) (Fig. 4). Post hoc analysis demonstrated that mRNA levels of PIK3CA (p110 α) were significantly elevated in UNP ($P = 0.023$) and UNPL ($P < 0.001$), but not UNL offspring, compared to Cont offspring (Fig. 4). Consistent with this, ovarian nuclear factor kappa light chain enhancer of B cells (NF κ B or RelA/p65) mRNA levels were significantly increased in ovaries from offspring born to undernourished mothers ($P < 0.001$) (Fig. 4). Post hoc analysis demonstrated an increase in NF κ B mRNA levels in UNP ($P = 0.002$), UNPL ($P < 0.001$), and UNL ($P = 0.017$) compared to Cont offspring (Fig. 4). Although offspring born to undernourished mothers tended to demonstrate increased mRNA levels of the proinflammatory cytokine IL-6, this difference was not statistically significant ($P = 0.063$) compared to Cont

FIG. 1. Early life exposure to undernutrition increases ovarian ER stress and follicular apoptosis but decreases ovarian autophagy in adult offspring. A) Maternal undernutrition during pregnancy and lactation (UNPL) resulted in an increase mRNA levels of ER stress-related genes expressed as a ratio of



XBP1s to XBP1t (n = 6–7 per group). **B**) Maternal undernutrition during pregnancy and during pregnancy and lactation resulted in an increase in follicular cell apoptosis (measured as TUNEL-positive staining) as a proportion of total area analyzed. **C**) Maternal undernutrition decreased key components of the autophagy process, including microtubule-associated protein 1A/1B-light chain 3 (LC3a) in UNP ovaries. **D**) Beclin 1 in ovaries of UNP and UNPL offspring. **E**) Photographs represent ovarian sections stained for immunopositive Beclin1 protein using DAB substrate and counterstained with hematoxylin. Immunopositive Beclin1 protein is identified by brown staining. Bars = 100 μ m. Negative controls show no positive staining (data not shown); n = 5 per group. **F**) Gene expression data are presented as means \pm SEM. One-way ANOVA main effects: maternal diet $P < 0.05$ for all genes. Post hoc analyses (Bonferroni): * $P < 0.05$ for undernourished compared to control and UNL offspring. Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone; n = 6–7 per group.

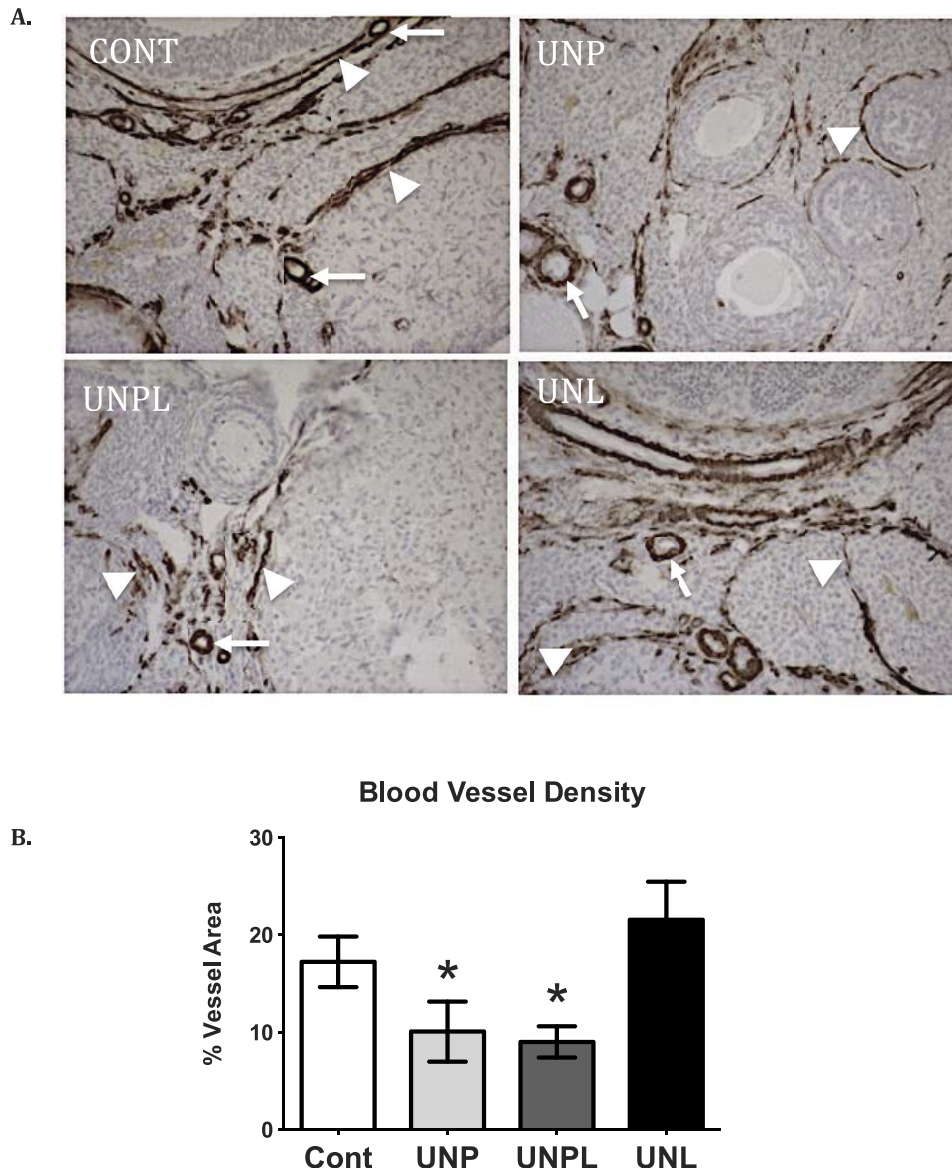


FIG. 2. Early life undernutrition reduces ovarian blood vessel density. Photographs represent ovarian sections immunostained for endothelial cell marker CD31 using DAB substrate and counterstained with hematoxylin. **A**) CD31 immunopositive protein is identified by brown staining indicative of DAB substrate. CD31-positive endothelial cell staining within ovarian blood vessel structures are indicated by white arrows. Perifollicular capillaries, that provide vascular supply to the developing follicle and/or corpus luteum, are indicated by white arrowheads. Negative controls show no positive staining (data not shown). Original magnification $\times 200$. **B**) Maternal undernutrition resulted in a significant decrease in the proportion of CD31 immunostaining (expressed as percent of vessel area of total ovarian area analyzed) in offspring ovaries. Data are presented as means \pm SEM. One-way ANOVA main effects: maternal diet $P < 0.05$ for undernourished compared to Cont offspring. Post hoc analyses (Bonferroni): * $P < 0.05$ compared to Cont offspring; $n = 5$ per group. Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone.

offspring (Fig. 4). Ovarian mRNA levels of IL-1 β , a proinflammatory factor shown to suppress apoptosis in the rodent ovary [50], was decreased in offspring born to undernourished mothers ($P < 0.001$). Post hoc analysis demonstrated that mRNA levels of IL-1 β were significantly decreased in UNP ($P < 0.001$) and UNPL ($P < 0.001$) but not UNL offspring compared to Cont (Fig. 4).

Maternal nutrient restriction also decreased mRNA levels of the gene encoding cyclin-dependent kinase inhibitor 1 (p21) (gene *CDKN1A*) ($P = 0.005$) (Fig. 4). Post hoc analysis demonstrated that this is largely attributable to a significant decrease in UNP offspring ($P = 0.049$) and not UNPL or UNL groups compared to Cont offspring (Fig. 4). This decrease is

consistent with our previously observed loss of ovarian follicles [13] and p21's role in inhibiting cell proliferation.

Early Life Undernutrition Exposure Reduced Ovarian Levels of the Melatonin Synthesizing Enzyme HIOMT

Melatonin, in addition to its role in circadian rhythmicity, is also a known antioxidant in the ovary [39]. Because we have previously shown that early life undernutrition resulted in increased ovarian oxidative stress levels [13], we investigated whether this may be associated with changes in the capacity of the ovary to produce melatonin. Early life undernutrition resulted in a significant decrease in HIOMT mRNA levels ($P = 0.002$) (Fig. 5). Post hoc analysis demonstrated that although

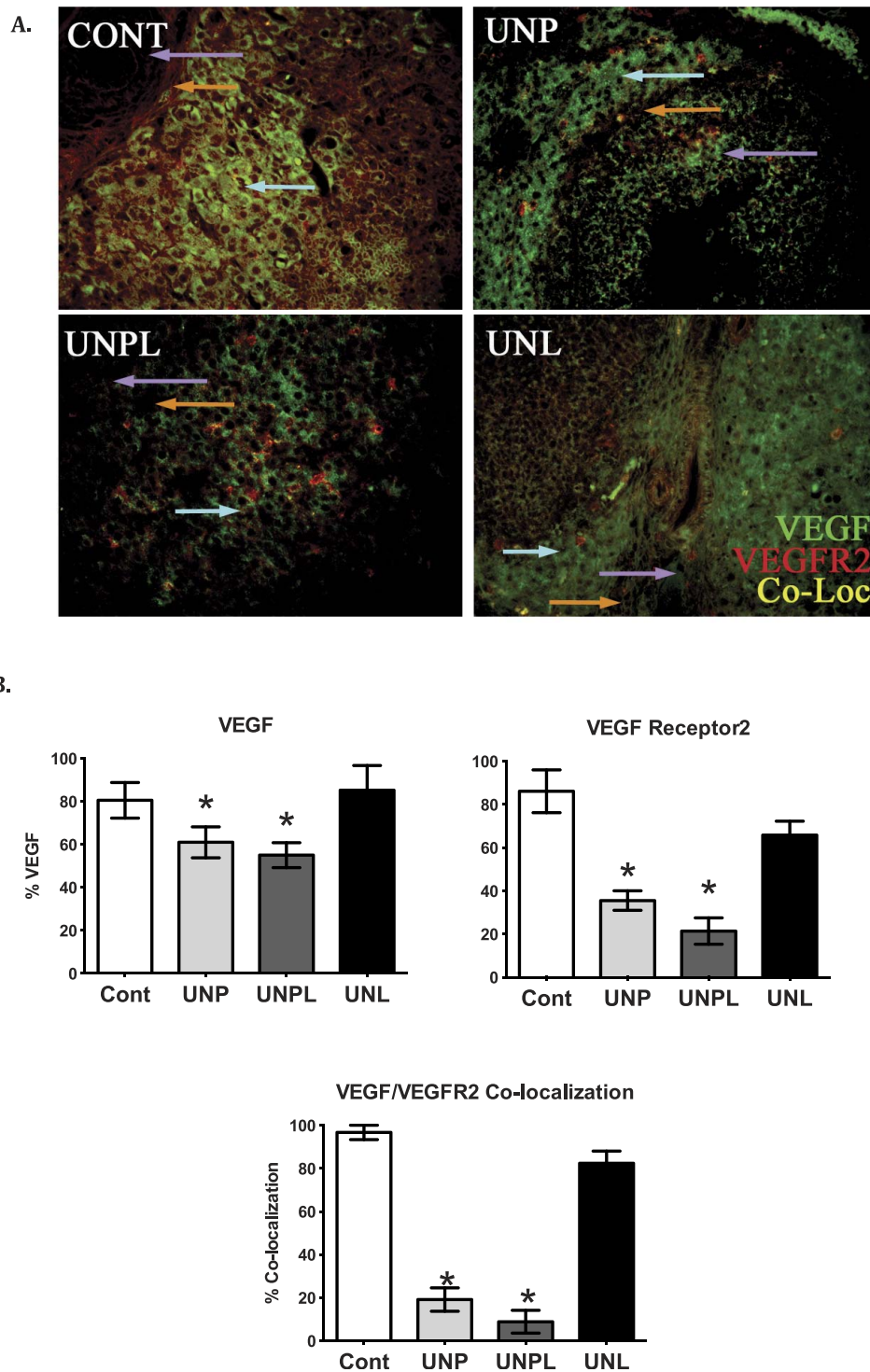


FIG. 3. Early life undernutrition reduces VEGF, VEGFR2, and their colocalization in offspring ovaries. **A)** Photographs represent immunopositive staining of VEGF (green), VEGFR2 (red), and the colocalization of VEGF/VEGFR2 (yellow). Negative controls show no positive staining (data not shown). Original magnification $\times 200$. **B)** Early life undernutrition resulted in a significant decrease in the proportion of VEGF, VEGFR2, and VEGF/VEGFR2 immunostaining (expressed as percent of positive stained area of total ovarian area analyzed) in offspring ovaries. One-way ANOVA main effects: maternal diet $P < 0.05$. Post hoc analyses (Bonferroni): $*P < 0.05$ for undernourished compared to control offspring ($n = 5$ per group). Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone. Cell types are indicated by colored arrows: granulosa cells (purple arrow), theca cells (orange arrow), and ovarian stroma (light blue arrow).

UNP offspring tended to have lower HIOMT mRNA levels, differences were significant only in UNPL ($P = 0.038$) offspring compared to Cont and UNL offspring (Fig. 5). We were unable to find detectable mRNA levels of the two known melatonin receptors in the ovary [51].

Early Life Undernutrition Exposure Altered Core Circadian Clock Genes in Offspring Ovaries

A close relationship exists between melatonin and clock genes [40, 52], and alterations in clock-controlled gene

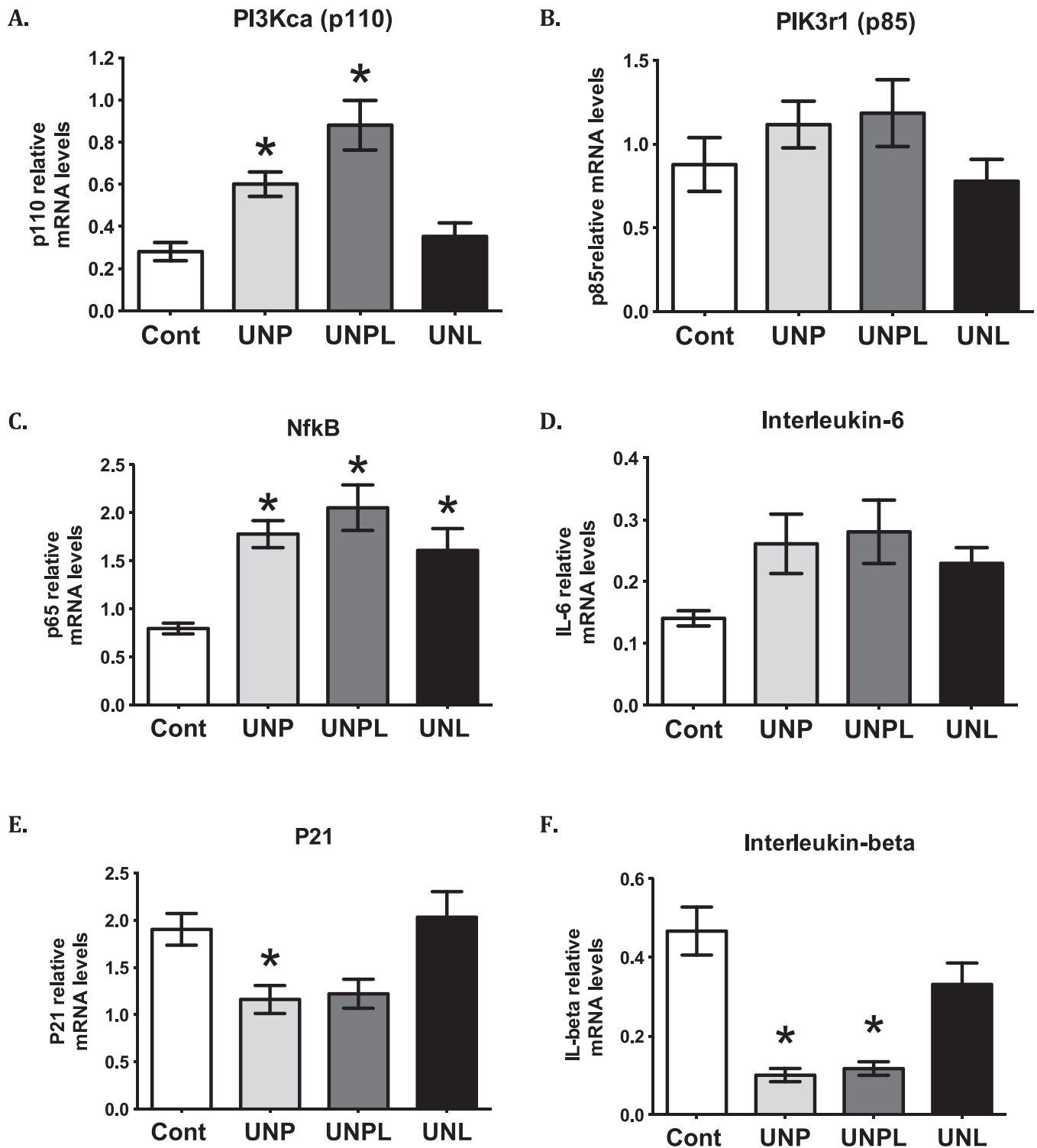


FIG. 4. Early life undernutrition alters proinflammatory cytokines and upstream signaling molecules in offspring ovaries. Early life undernutrition resulted in a significant increase in the catalytic subunit of PI3 kinase PI3KCA (A), p85 (B), and NFκB (C) mRNA levels, but a decrease in p21 (E) and proinflammatory IL-1β (F). (D) No effect on IL-6 was observed. Data are presented as means ± SEM. One-way ANOVA main effects: maternal diet $P < 0.05$ for all genes except for IL-6 undernourished compared to Cont offspring. Post hoc analyses (Bonferroni): * $P < 0.05$ compared to Cont offspring for PI3KCA and p21 and for IL-1β * $P < 0.05$ compared to Cont and as well when compared to UNL ($n = 6-7$ per group). Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone.

expression due to prenatal malnutrition have been linked to metabolic dysfunction in offspring later in life [53]. Clock gene knockout mice have illustrated the importance of clock genes in the regulation of reproductive function [54], and recently,

maternal obesity has been associated with altered clock gene expression in offspring ovaries [41]. Thus, we investigated the effect of early life undernutrition exposure on offspring ovarian mRNA levels of core circadian clock genes. Early life

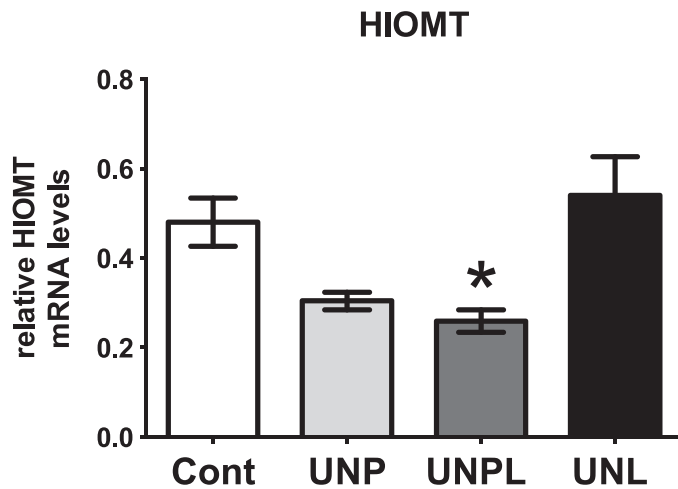


FIG. 5. Early life undernutrition alters ovarian levels of HIOMT. Early life undernutrition resulted in a significant decrease in ovarian mRNA expression of hydroxyindole *O*-methyltransferase (HIOMT) in a manner that is dependent upon the timing of nutrient restriction. Data are presented as means \pm SEM. One-way ANOVA main effect: maternal diet $P < 0.05$ for undernourished compared to Cont offspring. Post hoc analyses (Bonferroni): * $P < 0.05$ for UNPL ($P = 0.1$ UNP) compared to Cont and UNL offspring ($n = 6-7$ per group). Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone.

undernutrition resulted in a significant increase in CLOCK mRNA levels ($P = 0.015$). Post hoc analysis demonstrated an increase in ovarian Clock mRNA levels in UNPL ($P = 0.028$) and UNL ($P = 0.046$), but not UNP offspring compared to Cont offspring (Fig. 6). Although early life undernutrition tended to increase BMAL1 (also known as Arntl) mRNA levels, this difference was not statistically significant ($P = 0.081$). Ovarian cryptochrome 1 (CRY1) mRNA levels were significantly higher in undernourished offspring (main effect $P < 0.001$; Fig. 6). Post hoc analysis demonstrated that undernourished offspring showed higher ovarian CRY1 mRNA levels compared to Cont offspring, regardless of the timing of maternal undernutrition (UNP, $P < 0.001$; UNPL, $P = 0.004$; UNL, $P = 0.031$) (Fig. 6). There was no effect of early life undernutrition exposure on ovarian CRY2 mRNA levels ($P = 0.464$). Early life undernutrition exposure resulted in a significant increase in ovarian Period 1 (PER1) mRNA levels ($P = 0.033$) in offspring. Post hoc analysis demonstrated that this increase was largely due to an increase in ovarian PER1 mRNA levels in UNP ($P = 0.02$) compared to Cont offspring (Fig. 6); although UNPL and UNL levels tended to be higher, these differences were not statistically significant. There was a significant main effect of early life undernutrition on ovarian PER2 mRNA levels in offspring ($P = 0.025$), but post hoc analysis demonstrated no differences between groups. The mRNA levels of the transcriptional repressor Rev-erb α , involved in circadian rhythmicity, were not affected by early life undernutrition ($P = 0.1$) (data not shown).

DISCUSSION

Our data highlight the possible mechanisms that may be responsible for the postnatal ovarian phenotype characterized by premature ovarian aging after early life exposure to caloric restriction. We demonstrate that outcomes are dependent on the timing of nutrient restriction, where in most outcomes

measured, pregnancy is a critical time window of vulnerability as evidenced by the fact that UNP and UNPL offspring demonstrate the most significant changes. We show that offspring have increased follicular apoptosis and reduced blood vessel density that may be facilitated by a loss of VEGF and its receptor. We hypothesize that this ovarian phenotype may be mediated by increased ER stress and a loss of the capacity to produce the antioxidant melatonin. These observations are consistent with our previous observations whereby offspring that are exposed to early life undernutrition have a loss of follicles and demonstrate increased oxidative stress [13].

Our previously reported loss of follicles in undernourished offspring [13] could be mediated by ER stress-induced follicular apoptosis. Although we report only XBP1 as a marker of ER stress, it has previously been shown that many upstream unfolded protein response (UPR)-sensitive genes may converge on XBP1 transcription and mRNA splicing [55], and indeed XBP1 has been directly associated with disease states [56] and with autophagy [57]. Recent work has highlighted the role of ER stress-mediated UPR in granulosa cells [58]. Consistent with our observations, both ER stress and autophagy have been shown to be present in the goat ovary and regulate granulosa cell apoptosis [58]. Recent in vitro work shows that UPR signaling pathways that are activated by ER stress appears to play a role in luteal stage progression and ER stress-mediated apoptosis in induced corpus luteal regression [59]. It is possible that our observed increase in XBP1 ratio is due to corpus luteum regression, although it is likely that follicular UPR also contributes to our observation because these ovaries are within the follicular stage of the rat estrous cycle. [60, 61]

Antral follicle loss may occur through other possible molecular targets, including XIAP (X-linked inhibitor apoptosis protein). XIAP is localized within granulosa, theca, and luteal cells where it serves to regulate follicle loss through atresia [62, 63] and associates with survival signaling pathways, including PI3K, Akt, and NF κ B [64]. Importantly, ER stress induces transcription of IAPs [65] and interacts with the PI3K/Akt pathway where transcription of XIAP was up-regulated by PI3K/Akt, at least in vitro [66], to play a critical role in controlling cell survival by resisting ER stress-induced cell death signaling [66]. It is tempting to speculate that the survival-to-death balance within the ovary, particularly in growing (antral) follicle subtypes, has been tipped in undernourished offspring, whereby ER stress-triggered PI3K signaling is insufficient to restrain ER stress-induced apoptosis in undernourished offspring. Our previous observations that undernourished offspring have increased oxidative stress and reduced ability to cope with oxidative stress [13] is consistent with this notion.

It is important to recognize that PI3K signaling is a master regulator of diverse outcomes. In the ovary, PI3K signaling of Akt induces downstream effects that are not only dependent upon the cell type, (granulosa versus oocyte) but also the follicle subtype (primordial versus antral). Importantly, stimulation of PI3K/Akt leads to accelerated primordial recruitment in normal ovaries [67]. It is possible that early life exposure to undernutrition results in PI3K-induced accelerated primordial follicle recruitment; this may explain our previously reported loss of primordial follicle in UNPL offspring in adulthood [13]. Indeed early life exposure to high levels of testosterone (mimic of polycystic ovary syndrome) has been suggested to result in accelerated primordial follicle recruitment [68]. We would hypothesize that these recruited primordial follicles undergo atresia and contribute to a decrease in ovarian reserve [13]. We are unable to dissect out specific PI3K-Akt roles in whole ovarian

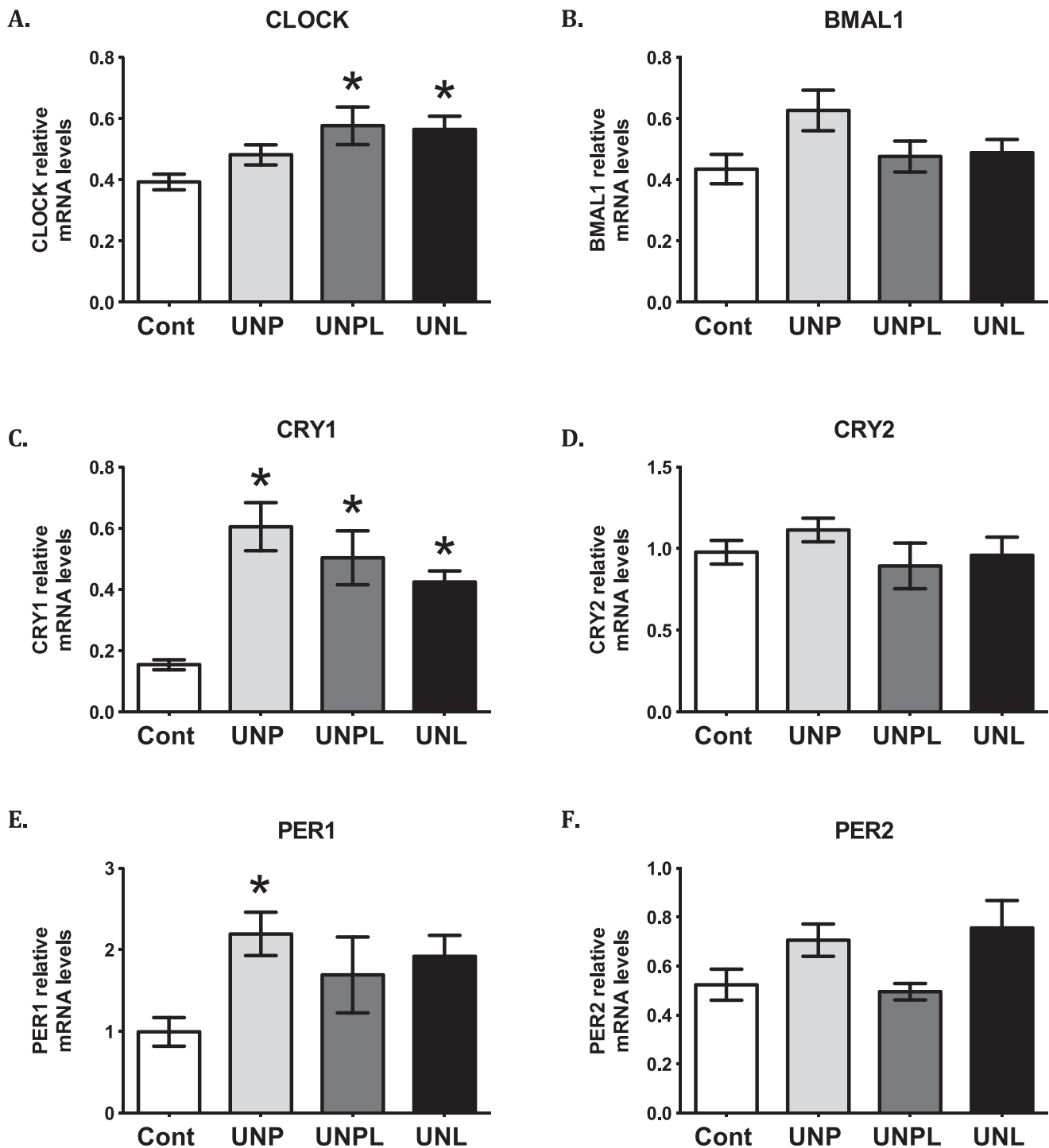


FIG. 6. Early life undernutrition alters core circadian clock genes in offspring ovaries. **A, B, C, E**) Early life undernutrition resulted in a significant increase in ovarian mRNA expression of core clock genes in the ovary in a manner that is dependent upon the timing of nutrient restriction: CLOCK (**A**), BMAL 1 (**B**), CRY1 (**C**), PER1 (**E**). Data are presented as means \pm SEM. One-way ANOVA main effects: maternal diet $P < 0.05$ for all genes except for PER2 (**F**) and CRY2 (**D**) levels in undernourished compared to Cont offspring. Post hoc analyses (Bonferroni): * $P < 0.05$ compared to Cont offspring ($n = 6-7$ per group). Cont, offspring of mothers fed a control diet; UNP, offspring of mothers undernourished during pregnancy alone; UNPL, offspring of mothers undernourished during pregnancy and lactation; UNL, offspring of mothers undernourished during lactation alone.

tissue, and as such, we recommend future studies engage in more culture-based queries or laser microdissection of specific follicles [69] to not only specifically pinpoint the exact downstream effect of an increase in PI3KCA (the catalytic subunit of PI3K) in UNP, UNL, and UNPL offspring but to investigate the role of PI3K in different follicle subtypes. Nevertheless, our data strongly implicate the PI3K pathway in

producing offspring ovarian impairment after early life undernutrition and in mediating our previously reported premature loss of ovarian reserve in adulthood [13].

Although our results suggest autophagy is not involved in the observed follicle loss in the UNP and UNPL adult offspring ovaries, previous studies have indicated that Beclin-independent pathways may also contribute toward the

activation of autophagy. However to date, this work has been shown in cortical neuronal cells, and ovarian carcinoma cells and the mechanisms by which Beclin-independent autophagy occurs varies according to cell type and stimuli [70, 71].

Proinflammatory cytokines are crucial in ovarian physiology; including ovulation, the maintenance of ovarian follicles, and regulating follicle atresia [50]. Proinflammatory IL-6 and its soluble receptor promote granulosa cell survival, regulating follicle growth [72]. IL-1 β has been shown to be a survival factor, influencing growth and preventing follicles from undergoing atresia by increasing nitric oxide production within granulosa cell and macrophages, inhibiting apoptosis [73]. Our observed reduction in IL-1 β mRNA levels suggests that UNP and UNPL offspring ovaries may be in a state of ER stress-induced apoptosis, aided by a decrease in IL-1 β . Interestingly, VEGF is induced by IL-1 β , which contributes toward perfollicular blood flow, ultimately promoting macrophage recruitment [74]. This is consistent with our observed loss of immunopositive VEGF and VEGFR2 in UNP and UNPL offspring, suggesting that local growth factors such as VEGF likely participate in impairments in folliculogenesis after early life undernutrition. Indeed, it has been previously shown that VEGF protects granulosa cells from atresia [33], promotes early folliculogenesis [75], and more recently is implicated in granulosa cell-oocyte cross talk [69]. Our observed loss of VEGF/VEGFR2 may be indicative of a loss of this protection from atresia.

Our study is the first to demonstrate that early life exposure to nutrient restriction alters adult offspring ovarian mRNA levels of core circadian genes and has associated changes in clock-controlled genes. Circadian clocks have been shown previously to exist in the ovary [76–78], and clock gene-null mice have delayed pubertal onset, altered estrus cyclicity, reduced ovarian weight [54, 79], reduced lifespan, and premature aging [80]. At the molecular level, clock genes, *Clock* and *Bmal1*, heterodimerize and result in the direct rhythmic transcription of *Per* and *Cry* gene families [81]. Our observation of increased *Clock*, *Per1*, and *Cry1* gene levels in undernourished offspring suggests that early life exposure to nutrient restriction may result in the resetting of the ovarian clock in undernourished offspring. Recently, a maternal high fat diet has also been shown to modulate core clock genes in offspring ovaries [41]. The heterodimer *CLOCK/Bmal1* controls the transcription of ~10% of the genes in a given cell; it binds to E-boxes that are located in the promoters of clock-controlled genes, including those that regulate steroidogenesis [82], apoptosis [83], cell cycle [84], inflammation [85], and angiogenesis [86], all putative regulators of ovarian folliculogenesis. How clock gene resetting modifies ovarian function is presently unknown, although a strong relationship exists between ovarian clock gene activation and gonadotropin regulation of follicle development [87]. It has been suggested that immature granulosa cells do not exhibit a strong circadian gene periodicity and that during the process of follicular differentiation, FSH provides a cue for the development of a functional clock [88]. We have previously reported that undernourished offspring have similar circulating FSH and FSH receptor levels compared to controls [13]. Thus, it is unclear how an increase in the core clock genes in UNPL (and modestly in UNP) offspring specifically affects follicle dynamics. We have, however, observed changes in ovarian steroidogenic capacity [13] and now show changes in apoptosis, cell cycle regulation, inflammation, and angiogenic pathways. It has been shown that follicular cells express cell autonomous circadian rhythmicity, which can be affected by gonadotropins FSH and LH [87], an effect that is dependent on

the cell's stage of differentiation. It is possible that clock gene signaling in FSH-dependent follicles may be compromised in these offspring, consistent with our previously observed loss of antral follicle number in undernourished offspring [13].

Melatonin has known effects on clock gene expression [40] and is a well-characterized antioxidant within the ovary [34, 35]. It induces ovarian cell survival [34] via reducing follicular atresia [34], improving in vitro fertilization rates [36] and oocyte quality [37]. We have shown that early life undernutrition decreases mRNA levels of the rate-limiting enzyme (HIOMT) involved in the production of melatonin. A decreased capacity to produce melatonin in UNP and UNPL offspring ovaries would be consistent with our observation that undernourished ovaries have increased oxidative stress [13] and follicular apoptosis. Moreover, it is possible that melatonin's regulation of oxidative stress could be through its known immune-modulatory actions via the NF κ B pathway [89]. Melatonin suppresses NF κ B signaling in many disease states [90]. We hypothesize that UNP and UNPL offspring have lower ovarian melatonin levels, which would be consistent with our observed increase in NF κ B mRNA levels, and would be consistent with a loss of antral follicles due to oxidative stress and apoptosis.

Although there has been some dispute about whether the ovary actually produces melatonin [28], our data together those of others [91] suggest that the ovary is capable of producing melatonin. Serum melatonin levels show seasonal and circadian variation and are related to reproduction [92], and there are many reports linking melatonin with reproductive outcome and ovarian function [93–98]. Melatonin has been localized in the human ovary [34]. Although we were unable to detect melatonin receptor mRNA levels in offspring ovaries, others have localized melatonin, its receptors, and synthesizing enzyme (HIOMT) to granulosa and theca cells in the rodent ovary using immunostaining [51, 91, 99], although their role in these cell types is not well established. It is likely that they are expressed at very low levels and thus could explain why we were unable to detect receptor presence in whole ovaries.

We recognize that our study is not without limitations. Our mRNA data represent whole ovarian homogenates and do not represent specific follicle subtypes. However, we consistently demonstrate that, even at the whole ovarian level, robust changes in mRNA levels and immunolocalization of key proteins and growth factors in offspring that were exposed to early life undernutrition. Further, although our tissue collection occurred within a strict 3-h time frame beginning at 0900, the clock gene data reflect only one time point within the 24-h circadian cycle. Given that we demonstrate such strong changes in both core clock genes and other key factors involved in folliculogenesis suggests that these changes are robust and reflective of real changes in gene expression within undernourished ovaries compared to controls. These changes are consistent with others that have collected ovarian tissue at one time point during the day and show changes in clock gene levels [41].

In conclusion, we present novel data that maternal undernutrition during pregnancy and/or lactation significantly increased ovarian ER stress in adult offspring that was accompanied by decreased ovarian VEGF, vascularization, and autophagy. It appears that a local ovarian antioxidant, melatonin, may be reduced because its synthesizing enzyme, HIOMT, is suppressed, a result that is consistent with our previous observations of increased ovarian oxidative stress in offspring of undernourished mothers [13]. We hypothesize that these changes may be downstream of clock gene expression patterns in the ovary. Critically, these results are dependent

upon the timing of maternal undernutrition, and the offspring whose undernutrition exposure included fetal life are particularly vulnerable.

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